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Comparative Studies on Neutralisation of Primary HIV-1 Isolates by Human Sera and Rabbit Anti-V3 Peptide Sera

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IgG binding to V3 peptides and serum neutralising responses were studied in four HIV-1 infected individuals with progressive disease over a period of 31–70 months. The 18–20 mer peptides comprised residues 299–317 (numbering of HIV1 MN) in the N-terminal half of the V3 loop of the envelope glycoprotein gp120 and were derived from the sequences of autologous, as well as heterologous isolates. All four individuals studied lacked anti-V3 IgG binding to at least one autologous V3 sequence. V3 peptides to which autologous sera lacked binding IgG were all immunogenic in rabbits and induced antisera that were broadly cross-reactive by EIA and broadly cross-neutralising to primary HIV-1 isolates. This indicates that the peptides are immunogenic per se and that the respective human hosts have selective defects in recognising the corresponding V3 sequences. Despite the absence of antibody binding to autologous V3 peptides, the human sera had neutralising antibodies to autologous (three out of four cases), as well as heterologous isolates (all cases). Moreover, in vitro exposure of the patients' isolates to autologous neutralising serum or the homologous rabbit antiserum selected for variants with amino acid substitutions close to the crown of the V3 loop or in regions outside the sequence corresponding to peptides used for immunisation. The amino acid exchanges affected V3 positions known to be antigenic and which are also prone to change successively in infected persons. It is likely that neutralising antibodies recognise both linear and conformational epitopes in the V3 loop. Apparently, there are several, but restricted, numbers of ways for this structure to change its conformation and thereby give rise to neutralisation resistant viruses. ***J. Med. Virol. 59:169–179, 1999.***

KEY WORDS: anti-V3 IgG peptide ELISA; neutralisation in vitro; V3 sequences

INTRODUCTION

The humoral immune response in HIV-1 infection is compatible with both viraemia and disease progression [Sawyer et al., 1990; Fenyö et al., 1996], and allows the establishment of a state of persistent infection. As a rule, neutralising antibodies appear several months after seroconversion and then have a narrow specificity [Albert et al., 1990; Koup et al., 1994]. In some individuals titres increase and specificity broadens over time and this is usually associated with slow, or no progression, to disease [Zhang et al., 1997]. Emergence of variant viruses resistant to neutralisation by autologous sera can however, be observed frequently, complicating further the picture [Albert et al., 1990; Von Gegerfeldt et al., 1991]. An understanding of this interaction of virus with the immune system is a prerequisite for the design of immunotherapeutic strategies [Haynes et al., 1996; Wyatt et al., 1998]. Several viral determinants of antibody mediated neutralisation of HIV-1 have been described. These are epitopes in the

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viral envelope, such as in the transmembrane glycoprotein gp41, the variable regions V1–V2 and V3, as well as those in the CD4 receptor binding region of the surface glycoprotein gp120 [Goudsmit et al., 1993; Wyatt et al., 1998]. The putative principal neutralisation determinant (PND) of HIV-1, situated in the V3 region [Javaherian et al., 1989; Vogel et al., 1994], also appears to be a critical determinant of chemokine receptor usage [Cocchi et al., 1996], cell tropism, and syncytium induction in cell lines [Milich et al., 1993]. During natural infection, intra-individual variation of V3 is characterised by successive changes restricted to particular amino acid positions, probably reflecting the functional and antigenic importance of those sites [Yamaguchi et al., 1997; Poss et al., 1998]. Yet one study demonstrated that a near total depletion of IgG antibody to the V3 region (measured in peptide EIA) from HIV-1 positive sera affected only minimally the capacities of the sera to neutralise primary isolates, thereby questioning the whole concept of V3 as an important epitope for neutralisation of HIV-1 in vivo [Vancott et al., 1995]. Nevertheless, there is a vast literature on V3 epitopes, which can either be broadly reactive [Javaherian et al., 1990; Blomberg et al., 1993; Cheingsong-Popov et al., 1994], or isolate specific [Boudet et al., 1996; Coeffier et al., 1997]. Isolate specific V3 epitopes are located mainly in the N-terminal half of the loop. This, and our initial observation of particularly prominent differences between HIV-1 positive sera in IgG binding to peptides from the N-terminal half of V3 [Lawoko et al., 1995], led to emphasis on the N-terminal V3 peptides in this study. Isolate specific defects were found previously in the anti-V3 IgG repertoire of some HIV-1 infected individuals when measured by EIAs using N-terminal V3 peptides [Lawoko et al., 1995]. If such serological patterns reflect virus neutralisation in vivo, then the defects could explain in part the failure of the humoral immune system to control HIV infection.

Among the questions raised by the initial findings were 1) whether the V3 peptides to which autologous IgG binding was lacking represent structures that are both antigenic and immunogenic, 2) whether antisera to the epitopes concerned can confer neutralisation of primary isolates that carry the corresponding epitope, 3) whether sera that contain the defects can neutralise autologous isolates. The earlier studies were limited by the fact that a single clone was sequenced from each individual. In the present study sera were examined from four HIV-1 infected individuals selected with isolate specific defects in their anti-V3 IgG repertoire. Synthetic peptides representing 2–4 variants covering the most prevalent proviral sequences in each individuals PBMCs, as well as a panel of V3 peptides from isolates described earlier were used in a longitudinal serological study. V3 peptides from each individual were used to immunise rabbits and obtain antisera. Neutralisation assays using such rabbit antisera, and autologous human sera were carried out. Finally, the variant viruses grown in vitro in the presence of neutralising antibodies were sequenced and the deduced amino acid sequences analysed. The results show that all V3 peptides to which the human host lacked IgG were immunogenic in rabbits and the epitopes concerned conferred neutralisation of primary HIV-1 isolates. Furthermore, data from the EIAs, neutralisation studies, and the analysis of antibody selected V3 sequences converged to show the importance of certain residues for V3 loop antigenicity and neutralisation.

MATERIALS AND METHODS

Patients and Sera

Sera were obtained sequentially from patients attending the infectious disease clinics of the central hospitals in Helsingborg and Lund over a period of 31–70 months. Early serum used in neutralisation assays was in all cases obtained simultaneously with the HIV-1 isolate. Serology of sera from two individuals, SP1 and SP2, served to establish the phenomenon of defects in the anti-V3 repertoire [Lawoko et al., 1995]. These studies have been extended and include samples from two additional patients (FP1 and FP2). The exact time of infection of the four patients was not known, but was estimated on indirect clinical grounds to be 3–4 years prior to the beginning of the study (the first serum and PBMC sample). Two broadly cross-reactive sera, a pool of 15 HIV+ sera named "Pool 7" and a single serum from an individual identified as "1785" [Weber et al., 1996], were included in the neutralisation assays for comparison.

Signs and Symptoms

Each of the following clinical and laboratory findings was given one point: Oral thrush, hairy leucoplacia, herpes zoster, ulcerative herpes simplex, seborrheic dermatitis, ungular mycosis, thrombocytopenia ($<100 \times 10^9$/l), leucopenia ($<3 \times 10^9$/l), anemia (<110 g/l), IgG levels (>15 g/l), chronic lymphadenopathy >1 cm in two or more extrainguinal groups, chronic diarrhoea, >10% weight loss under a maximum of 3 months, and fever (>38°C) for longer than 1 month. Every new sign or symptom was then added to give a total number of signs and symptoms (TNSS) at different time points of the follow-up. AIDS diagnosed according to the Walter Reed criteria [Redfield et al., 1986] was entered as ten points, the maximum number given. Opportunistic infections such as pneumocystis C. pneumonia, toxoplasmosis, atypical mycobacteria also led to ten points, as did HIV associated malignancies and dementia. This enumeration is used only as an ad hoc measure of disease severity and a convenient way of including minor routine clinical observations which do not occur in established scoring systems, like in the Walter Reed or CDC staging systems [Redfield et al., 1986; MMWR 1992].

IgG EIA

EIAs were done as described earlier [Blomberg et al., 1983; Klasse et al., 1988]. Bound antibody was detected using alkaline phosphatase-conjugated antihuman IgG

(Orion Diagnostica, Espoo, Finland) and p-orthophenyl disodium phosphate substrate dissolved in diethylanolamine buffer. A peptide concentration of 20 μg/ml (2 μg/well) was used. Chosen after optimisation experiments, these conditions were found to give maximal binding of all peptides [Klasse et al., 1988; Lawoko et al., 1995]. Sera were tested at a dilution of 1/50. Absorbance differences were all standardised against the reactivity of a pool of HIV-1 positive sera (pool 7) which was examined simultaneously for each peptide. A cutoff value of 0.3 was set for positive reactivity. This corresponds to 10 standard deviations over the average peptide reactivities of 69 seronegative blood donor sera. As a control for the degree of non-HIV antiviral IgG binding in the EIAs influenza A H3N2 and measles antigens were used, as well as uninfected cells prepared similarly as negative antigen controls. These antigens, purchased from the National Bacteriological Laboratory in Sweden, were tested by EIA experiments under the same conditions as described for synthetic peptides. The concentration of the whole viral antigens was optimised by checkerboard titration to encompass the whole range of IgG binding encountered in normal individuals, utilising the experience from routine diagnostic work in the laboratory. To enable a compact presentation of salient features, binding data were presented as strong, intermediate (weak) or negative, reflecting the intensities of optical density.

IgG Purification

IgG for use in neutralisation assays was purified from sera using HiTrap Protein G affinity columns (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers instructions. The concentration of purified IgG was adjusted with that of the corresponding serum before use in neutralisation assays.

Synthetic Peptides

The primary sequences of peptides were determined from the nucleotide sequences of proviral DNA in primary isolation cultures, as well as from earlier studies [Myers et al., 1996]. Solid phase synthesis was carried out using an automated multiple peptide synthesiser (AMS 422. Abimed Analysen-Technik, Langenfeld, Germany) [Gausepohl et al., 1992]. Tetrameric peptides based on a backbone of β-alanine and two lysines were synthesised for rabbit immunisations. The peptides were purified by reverse-phase high performance liquid chromatography (HPLC) on a C18 column to over 95% purity. Sequence alignment and similarity scoring was done using an earlier published method [Dayhoff et al., 1983]. Except for the full length MN V3 and the gp41 peptide, 18-20 mer peptides encompassing the residues 299–317 were used in the study. Unless stated otherwise, amino acid positions were named according to HIV-1 MN (GenBank accession number M17449). The gp41 peptide was included as a non-V3 HIV peptide control.

Virus Isolation

Patient PBMCs, separated from 5–10 ml EDTA-blood by Ficoll-paque (Pharmacia, Uppsala, Sweden) centrifugation, were cocultivated with PHA-stimulated (2.5 μg/ml for 3 days) PBMCs from two blood donors [Global program on AIDS (WHO) 1994]. RPMI 1640 supplemented with 10% fetal calf serum (Life Technologies, GIBCO BRL, Grand Island, NY), 5 units/ml recombinant IL-2 (Boehringer Mannheim, Germany), 2 mg/ml polybrene (Sigma, St. Louis, MO), penicillin, and streptomycin was used for culture maintenance. Fresh PBMCs from two seronegative blood donors were added to the culture weekly and HIV-1 p24 antigen production was tested every 3–4 days over a period of 4 weeks. DNA from PBMCs in antigen positive cultures was purified for PCR amplification, cloning, and sequencing of the V3 loop region.

Virus Neutralisation Assays

Neutralisation assays were carried out as described earlier [Albert et al., 1990] with a readout of reverse transcriptase activity [Ekstrand et al., 1996]. Briefly, an intended dose of 2 ^{10}log $TCID_{50}$ of virus was used to inoculate 100,000 PHA stimulated PBMCs/well on 96-well microtitre culture plates. In individual experiments an actual virus dose of 1.41 ± 0.25 (s.e.m.) ^{10}log was obtained. The importance of this variation was small, because the most critical comparisons, between the number of V3 clones obtained before and after neutralisation, and between patient serum and the corresponding purified total serum IgG, were carried out in the same experiment with exactly the same viral dose. Two-fold dilutions of sera (range: 1:50–1:800 for rabbit sera and 1:20–1:320 for whole human sera or IgG preparations) were used. Cultures were washed twice (days 2 and 4) and were maintained until day 7 when reverse transcriptase activity in supernatants was measured. The highest serum dilution giving a 90% reduction in reverse transcriptase activity compared to that in cultures not pre-incubated with serum was considered as the neutralisation titre. Plates were then frozen at −80°C until the next day when samples for RNA preparation were selected.

RNA Preparation

Microtitre plates containing neutralisation experiments were thawed and centrifuged at 1,200 rpm for 10 minutes, before 50 μl of supernatant was harvested from relevant wells. Virus particles in culture supernatants were lysed in 0.1 M TrisHCl buffer containing GuSCN/EDTA/Triton at a pH of 6.4, after which RNA was bound to a silica matrix and washed [Boom et al., 1990]. All RNA samples were reverse transcribed into cDNA before PCR amplification.

DNA Amplification, Cloning, and Sequencing

Proviral DNA and cDNA were amplified by nested PCR using the outer primers (5′CACAGTACAATGTACACATG, 5′JA12-5′ ACAGTAGAAAAATTCCCCTC)

and inner primers (5′ AAATGGCAGTCTAGCAGAAG, 5′ ACAATTTCTGGGTCCCCTCC) covering the V3 region between the nucleotides 7191 and 7513 of the envelope gene of HIV-1 [Albert et al., 1990]. Amplified DNA was cloned (TA cloning kit, Invitrogen, San Diego, CA) and then sequenced using an automated laser fluorescence device (ABI Prism Dye Terminator Cycle Sequencer 31;0 Perkin-Elmer, Oak Brook, IL). A south Swedish consensus V3 sequence was obtained by the alignment of thirteen subtype B V3 sequences from southern Sweden [Lawoko et al., 1995].

Rabbit Immunisations

Multibranched (tetrameric) V3 peptides were dissolved in normal saline and the solution mixed with an equal volume of Freunds incomplete adjuvant, to give a final peptide concentration of 0.1 mg/ml; 1 ml of the mixture was used in subcutaneous inoculations of animals. Preimmune sera were obtained from each animal prior to the first inoculation. Booster immunisations were given after 2 and 4 weeks. Hyperimmune sera were obtained on weeks 4 and 6. Except for V3 peptides with sequences derived from the primary isolate of individual SP2 where two were used, a single peptide selected at random representing the proviral DNA sequence for each patient was included in the immunisation experiments.

Statistical Analysis

Fisher's exact test was used to study the relative frequency of variants in populations of isolates cultured under various neutralising conditions.

RESULTS

Binding of IgG in Human Sera to Autologous and Heterologous Peptides

Thirty-one V3 peptides (Fig. 1a) were tested with sera from four patients. Two patients could be classified as slow progressors (SP1 and SP2) and two as fast progressors (FP1 and FP2) on the basis of symptoms and CD4+ T cell decline [Scarlatti et al., 1996] (Fig. 1b). IgG in sera collected sequentially from three individuals (SP1, FP1, and FP2) lacked the capacity to bind autologous N-terminal V3 peptides in EIA throughout the 31–70 month follow-up. The slow progressor SP2 was exceptional in that peptides representing three variants of autologous V3 sequence were synthesised and tested. One, a minority sequence, did not react with autologous sera, while the other two did react. Considering all peptide reactivities, IgG in sera from the fast progressors FP1 and FP2 bound to fewer V3 peptides (4/31 and 12/31, respectively) compared with serum IgG from SP1 and SP2 (20/31 and 24/31, respectively) (Fig. 1b). Sera from patient FP1 exhibited a near total lack of IgG reactivity with all V3 peptides tested including the full length MNV3 (Fig. 1b). Total serum IgG levels were however normal (20–24 mg/ml) in all sera from FP1. Furthermore, influenza and measles specific IgG were measurable and virtually unchanged during the follow-up (data not shown), and IgG binding

a

Origin:	Name:	Amino acid sequence:
S.Swedish cons.	SSC	INCTRPNN--TRK-SIHI--GPG
SP1	SP1V3N1	......S.N.......P......
	SP1V3N2	.......KN.......P......
SP2	SP2V3N1	........N.......N......
	SP2V3N2	........K.......N......
	SP2V3N3	..............RMTM.....
FP1	FP1V3N1	......S.N...R.G..M.....
	FP1V3N2	......S.....R.G........
FP2	FP2V3N1	......D.N.......R.QR...
	FP2V3N2	................R.QR...
	FP2V3N3	........N.......R.QR...
S.Swedish V3	LU20V3N	....................A..
" "	LU31V3N	............R..........
" "	LU12V3N	.................M.....
" "	LU11V3N	...I...................
" "	HB21V3N	..............G........
" "	LU14V3N	.V..............R......
" "	HB22V3N	.......KN..............
Ugandan V3 (21)	UG72V3N	........N.....G.Y......
Ethiopian V3 (20)	ET2222V3N	........N......MR......
Zairean V3	Z321V3N	...M....N.......S......
S.Swedish V3	LU6V3N	.I....S.......G........
" "	LU25V3N	.H.I............YM.....
HIV1MN	MNV3N	.......YN.K...R........
HIV1MAL	MALV3N	......G.N...R.G..F.....
Ugandan V3 (21)	UG30V3N	......Y.N..-RQ.T.......
" "	UG65V3N	......Y-.KSI-.-RR......
S.Swedish V3	LU8V3N	.......-.KIKVR.M.......
HIV1Z3 V3	Z3V3N	......GSDKKIRQ..R......
Ugandan V3 (21)	UG16V3N	......YKNI.I-QRTP....L.
HIV1JY1	JY1V3N	......D.KI..-Q.TP....L.
HIV1MNV3FULL	CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC	
HIV1HXB2GP41	CTTAVPWNASWSNKSLEQIWNH(604-625)	

Fig. 1. **a:** V3 peptides segregated into autologous (top four boxes beneath the consensus SCC) and heterologous (lower subset), listed in order of decreasing similarity to a south Swedish consensus V3 sequence from top to bottom. Autologous peptides have sequences derived from the N-terminal halves of V3 loops from the primary isolates of patients. The full-length sequence of MN V3 and that of the control peptide from gp41 are given in the lowest box. References are indicated in brackets beside the origin. **b:** Single character presentation of data from longitudinal serology using synthetic V3 peptides and sera from two slow progressors (SP) and two fast progressors (FP). The timepoints in months (MO) for obtaining serum samples are listed vertically in the left column. "*", the timepoint of obtaining virus isolate from which the V3 sequences of the autologous peptides were derived; #, timepoint when "late sera" were obtained. Synthetic peptides are arranged horizontally in order of decreasing similarity to a south Swedish consensus V3 sequence, from right to left. IgG binding to V3 peptides: white for absorbance difference less than 0.3; grey for 0.3–1.0; and black for greater than 1.0. Samples not tested are represented by a dot. The letter "A" indicates binding to autologous peptide. The total number of symptoms and signs (TNSS), CD4+ lymphocyte count, and p24 antigenemia (AGQ) are also presented. TNSS were counted as described in materials and methods. Antigen quantity: white < 10 pg/ml; grey 10–15 pg/ml; black > 15 pg/ml. CD4+ cell count/μl: > 400 black; 200–400 grey; <200 white.

to the non-V3 HIV-1 control peptide (HXB2GP41) was also measurable throughout the follow-up (Fig. 1b). Thus, the lack of IgG binding to autologous V3 peptides was part of a general absence of anti-V3 IgG antibodies, while IgG of other specificities were not depleted. In sera of patient FP2 all IgG reactivity documented previously with the nine heterologous V3 peptides was lost upon, or just prior to, developing AIDS (Fig. 1b). Also in this case, other IgG antibodies exemplified by total IgG, anti-gp41 peptide, and influenza and measles specific IgG (data not shown), were not depleted.

The structural requirements for binding of IgG to N-terminal V3 peptides were examined in one case (in-

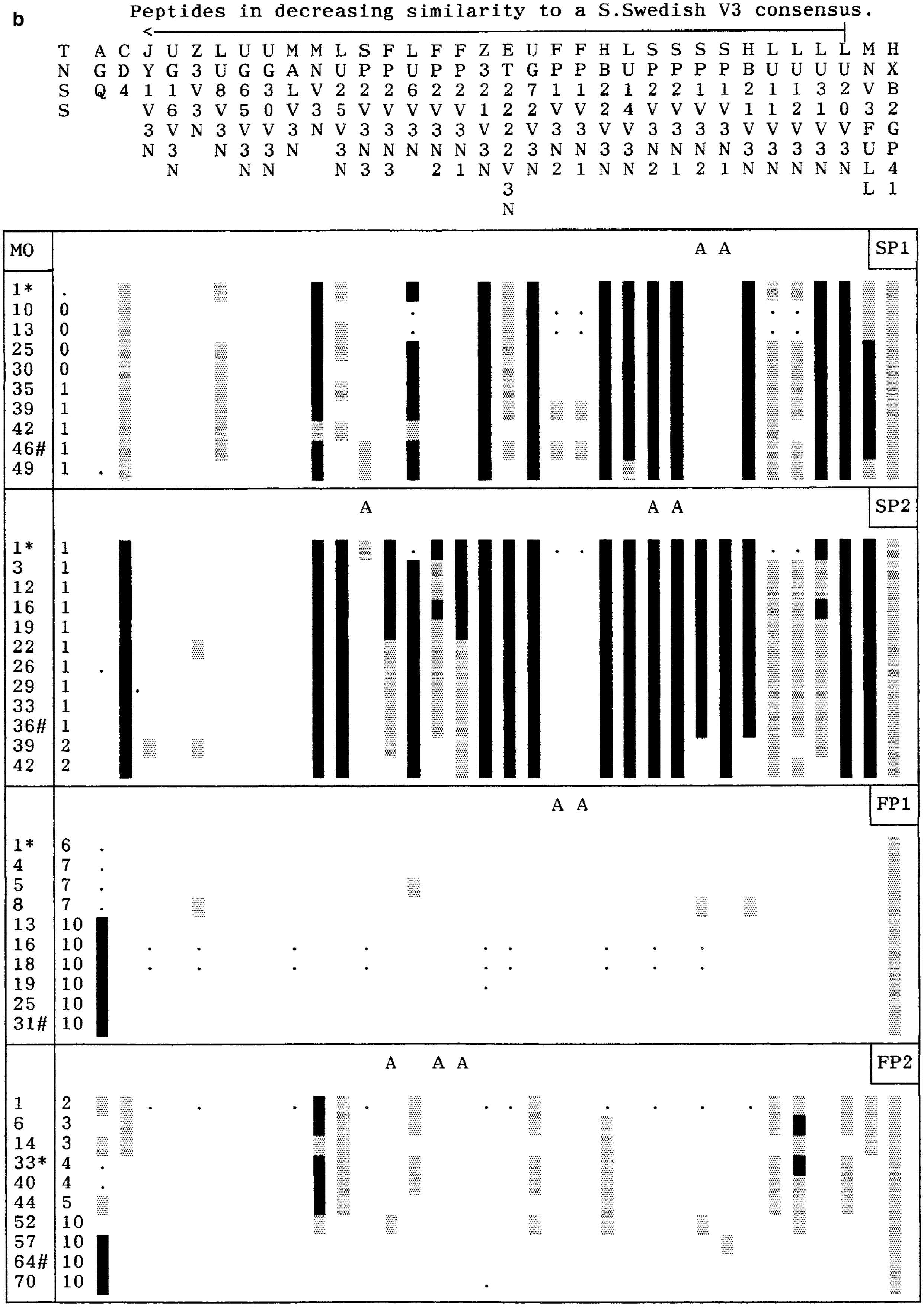

Figure 1 (continued).

V3 peptide	Amino acid Sequence	Serum sample time point: 1 10 13 25 30 35 39 42 46 49
SP1V3N2	INCTRPNKNTRKSIPIGPG 299-317	
SP2V3N2	.......NK.....N....	
variant 1	........K.....N....	
variant 2	..............N....	
variant 3	.......N......N....	
variant 4	........K..........	
variant 5	.......NK..........	
variant 6	.......N...........	

Fig. 2. Single character presentation of the binding of IgG in sera from individual SP1 to V3 peptide variants representing all possible substitutions of amino acids differentiating the non-reactive autologous peptide SP1V3N2 and the reactive heterologous one SP2V3N2. Peptides are listed on the vertical scale. Variants 1–6 have single amino acid substitutions. Sera are named according to the timepoint (in months) of sampling and are listed horizontally above the absorbance differences. Absorbance differences: white < 0.3; grey > 0.3–1.0; and black > 1.0. A proline at position 313 (V3 position 13) always abrogated IgG binding.

dividual SP1). IgG binding to both autologous V3 peptides SP1V3N1 and SP1V3N2 were not detected in sera from this patient, but IgG binding to several heterologous V3 peptides with sequences closely similar to autologous peptides was observed. This pattern, which was sustained over the follow-up period of 49 months, prompted a detailed study of the structural requirements for V3 peptide binding by IgG in sera from SP1. Using the sequence similarity to peptide SP2V3N2 (a difference of three residues, Fig. 1a), a series of substitution peptides were synthesised and used in serology with sequential sera from patient SP1. The replacement of asparagine by proline at position 313 (N $\rightarrow$ P) abrogated IgG binding to V3 peptides (Figure 2).

Neutralisation of Primary HIV-1 Isolates by Autologous and Heterologous Human Sera

In three out of four cases autologous serum collected at the time of virus isolation neutralised the corresponding isolate (sera SP1-E, SP2-E, and FP2-E and isolates SP1-PI, SP2-PI, and FP2-PI, respectively) (Table I). Neutralising titres either remained the same or were increased in the late autologous sera. In the heterologous reaction, early sera from all four patients neutralised at least one virus. Heterologous neutralising titres were higher [80–320] than autologous titres [20–80]. An early serum (FP1-E) from individual FP1, which had no detectable IgG binding to V3 peptides, did not neutralise the autologous isolate FP1-PI but all three heterologous isolates.

In order to determine the role of virus specific IgG responses in the neutralisation of primary isolates, whole serum was tested in parallel with the corresponding IgG fraction. The serum that was shown previously to neutralise strongly a given isolate (Table I) was chosen for these experiments. Purified IgG and whole serum neutralised isolates at titres that were usually similar (Table II). This indicates that the neutralising activity directed against HIV-1 primary isolates resides in the IgG fraction of human serum.

Virus Cultures in the Presence of Autologous Neutralising Sera

The V3 sequences of virus variants growing after 90% serum neutralisation were compared with the V3 sequences of viruses in cultures not exposed to serum (Fig. 3a,b). Proviral V3 sequences in patient/blood donor PBMC cocultures were also included in the comparisons, which were focused mainly on residues immediately flanking the crown of the V3 loop since they were previously found to be important for antigenicity of V3 peptides [Lawoko et al., 1995]. The culture of isolate SP1-PI exposed to neutralising autologous serum resulted in the emergence of a dominant variant different from the major fraction in a culture not exposed to serum. Unlike the majority sequence in the latter culture, which contained a histidine at position 313, the emerging variant contained a proline at this position (H $\rightarrow$ P) (Fig. 3a). The frequency of this proline containing variant was 15/46 before and 10/11 after neutralisation by autologous serum (P = 0.045), a moderate shift but unlikely to have occurred by chance. The frequency of sequences containing histidine at position 313 were 23/46 before, and 0/11 after neutralisation (P = 0.017). This change was particularly interesting because patient SP1 lacked IgG binding to V3 peptides with a proline residue at this position (Fig. 2). Culture of isolate SP2-PI exposed to neutralising autologous serum SP2-E resulted in the predominance of a V3 variant that formed a minor fraction in a culture not exposed to serum (Fig. 3a). This selection was concomitant with the replacement of glutamic acid by glycine at position 325 (E $\rightarrow$ G) in the C-terminal half of the V3 loop (frequency 1/50 before, and 10/10 after autologous serum neutralisation, P < 0.0001). The dominant sequence remained the same whether isolate

TABLE I. Neutralisation of Primary Isolates SP1-PI, SP2-PI, FP1-PI, and FP2-PI by Autologous and Heterologous Sera*

Isolate	Infectious dose ($TCID_{50}$)	SP1-E	FP2-E	FP1-E	SP2-E	Late autologous sera	POOL 7	1785
SP1-PI	32	20	80	160	0	20	80	320
FP2-PI	11.2	320	80	40	40	320	160	320
FP1-PI	37	80	0	0	0	0	40	20
SP2-PI	41	80	40	80	40	80	80	320

*Neutralisation assays using early (SP1-E, SP2-E, FP1-E, and FP2-E) and late sera with four primary isolates. The primary isolates used in these experiments consist of cell free virions obtained from patient/blood donor PBMC cocultures. Autologous combinations are identifiable from the names (e.g., isolate SP1-PI, with early serum SP1-E, both obtained from individual SP1) and their titres are boxed. Two broadly neutralising sera; Pool 7 and 1,785 were also included.

TABLE II. Neutralisation of Primary Isolates SP1-PI, SP2-PI, FP1-PI, and FP2-PI by Autologous Serum and Total Serum IgG*

Isolate	Infectious dose ($TCID_{50}$)	SP1-E (10 mg/ml)		FP1-E (20 mg/ml)		Pool 7 (21.8 mg/ml)	
		Serum	IgG	Serum	IgG	Serum	IgG
SP1-PI	32	ND		160	160	80	80
FP2-PI	11.2	320	110	ND		320	320
FP1-PI	37	160	160	ND		320	320
SP2-PI	41	160	80	ND		320	160

*The serum which had earlier been found to have the highest neutralising titre against an isolate was chosen for these experiments. The left column contains the isolate name. For each serum, the neutralising titres of whole serum and that of the total IgG counterpart are given beside one another. The concentration of total IgG in whole serum is given in brackets. Combinations not done are marked "ND." After purification, IgG concentration in culture medium was adjusted with that of the pre-determined total serum IgG before use in the assays.

FP2-PI was exposed to autologous serum neutralisation or not. However, in the serum exposed culture a variant emerged with several substitutions in both the C- and N-terminals and a deletion of the glutamine and arginine in positions 308 and 309 (-SIRI*QR*GPG-$^{304–312}$ based on the HIV-1 IIIB V3 clone NL4-3 sequence to which it is similar [Salminen et al., 1995]) (Fig. 3b). The frequency of this variant was 0/48 in cultures not exposed to serum and 2/10 in cultures subjected to neutralising autologous serum FP2-E ($P = 0.037$). Serum FP1-E from the fourth individual FP1 had no neutralising activity against the autologous isolate FP1-PI. Although a higher number of clones would have given a better statistical basis, the likelihood that the shifts in proportions observed in three out of three cases occurred by chance is small. The results suggest that autologous neutralising antibody exerts a selective effect when added to virus cultures in vitro.

Neutralisation of Primary HIV-1 Isolates by V3 Specific Rabbit Antisera

Tetrameric V3 peptides with sequences corresponding to the virus isolates of the four patients were all immunogenic in rabbits. The resulting antisera were all broadly cross-reactive by EIA (absorbance differences >0.6, results not shown) with the V3 peptides used for immunisation. This was in sharp contrast to the restricted pattern of binding seen with autologous human sera (Fig. 1b). The rabbit antisera were also broadly cross-neutralising to primary isolates (Table III). In four out of five cases the most potent neutralisation observed (titre range 200–800) was directed at a primary isolate from which the sequence of the immunising peptide originated (homologous neutralisation). However, neutralisation of at least two heterologous isolates was also observed. One exception was the antiserum A-SP2V3N3 raised to an aberrant, low frequency sequence present in isolate SP2-PI, which lacked neutralising activity against the homologous isolate. Preimmune rabbit sera did not neutralize any of the HIV-1 isolates. Thus, immunisation of rabbits with tetrameric N-terminal V3 peptides derived from a single sequence can induce immune responses that are broadly cross-reactive in EIA and also cross-neutralising to primary isolates.

Virus Cultures Following Exposure to Neutralising Rabbit Anti-V3 Sera

The broad cross-neutralising activity of rabbit anti-V3 sera prompted the analysis of the quasispecies present in virus cultures following neutralisation by such sera. When a culture of isolate SP1-PI was exposed to neutralising rabbit antiserum A-SPIV3N2 raised against peptide SP1V3N2 (containing proline at position 313; -SI*PI*GPG-), the main V3 sequence retrieved was the same as that in cultures not exposed to serum. It contained a histidine at position 313 (-SI*HI*GPG-) (Fig. 3a). There however, emerged a new variant with the replacement of glycine by arginine (G → R) at the usually highly conserved position 315 (-SIP*IR*PG-) within the antibody target sequence in the crown of the V3 loop. The frequency of this variant was 0/46 before,

a

Culture	V3 sequences	# of clones
SP1-PI	CTRPSNNTRKSIHIGPGRAFYTTGEVTGDIRQAHC$^{301-335}$	20
Without	............P......................	13
Serum	............N...........AI.........	5
	....N.......N.......FA...II........	2
	....G..............................	1
	............P...................HN.	1
	............P....................L.	1
	........................A..........	1
	............R......................	1
	..................................Y	1
		/46
SP1-PI	............P......................	4
Proviral	....NK......P....K...A....I........	1
DNA		**/5**
SP1-PI	............P......................	8
+	............P.....................R	1
Aut.	............P.-....................	1
Serum	............N...........AI.........	1
		/11
SP1-PI	....	6
+	............P.R....................	3
rabbit	..............R....................	1
serum		**/10**
SP2-PI	CTRPNNNTRKSINIGPGRAFFATGEIIGDIRQAHC	43
Without	...........M.......................	2
serum	.................................R.	1
	.........................T.........	1
	..K.........................Y......	1
	...............L...................	1
	........................G..........	1
		/50
SP2-PI	....	2
Proviral	......K.................G..........	1
DNA	......-...RMTM...KVYYT..Q.-....R...	1
	........................G..........	1
		/5
SP2-PI	........................G..........	9
+	..................V.....G..........	1
Aut.		**/10**
Serum		
SP2-PI	........................D..........	10
+		**/10**
rabbit		
serum		

b

Culture	V3 sequences	# of clones
FP1-PI	CTRPSNNTRRGIHMGPGRAFYTTGQIIGDIRQAHC$^{301-335}$	34
Without	....................F..............	8
serum	.....Y..............F..............	1
	.........K..........F..............	1
	............................N......	1
	..................H.F-NRTNNRRYKAS..	1
	...................L...............	1
	.....D.............................	1
		/48
FP1-PI	....	2
Proviral	......-......I............-.H.G....	1
DNA	....................F..............	1
		/4
FP1-PI	No autologous neutralization.	
+		
Aut.		
Serum		
FP1-PI		
+	ND	
rabbit		
serum		
FP2-PI	CTRPNNNTRKSIRIQRGPGRAFVTIGKI-GNMRQAHC$^{294-329}$	32
Without	........................M............	6
serum	......D..............................	2
	...................I.................	2
	...................S.................	1
	...................................P.	1
	........................KE.......R...	1
	...........................M.........	1
	.........................R...........	1
	......S..............................	1
		/48
FP2-PI	....	3
Proviral	......-..................-...........	1
DNA	........................M............	1
	....D................................	1
		/6
FP2-PI	....	8
+	.....Y.K..R.H.--......Y.TKN.I.TI.....	2
Aut.		**/10**
Serum		
FP1-PI	ND	
+		
rabbit		
serum		

Fig. 3. **a, b:** Amino acid sequences of V3 loops derived from viruses obtained after each isolate was subjected to different in vitro culture conditions. A dot and dash indicate similarity and deletion respectively. Isolates are named after the individuals from which they were obtained. Thus, SP1-PI for the primary isolate of slow progressor SP1 (a) and FP1-P1 for that from fast progressor FP1 (b). For all isolates, the sequences of virus in cultures not subjected to serum, cultures subjected to autologous serum, and of proviral DNA from PBMC co-cultures were studied. Variants of SP1-PI and SP2-PI obtained from cultures subjected to rabbit anti-V3 sera were also studied. The culture conditions under which given sets of sequences were obtained are given in the first column. Autologous serum cultures are labelled "Aut. Serum." Apart from sequences in FP2-PI, residues are numbered according to the primary sequence of HIV-1 MN. FP2-PI sequences are numbered according to HIV-1 IIIB clone NL4-3. The N-terminal sequences of peptides used to raise rabbit anti-V3 serum utilised in the neutralisation experiments are underlined in the proviral compartment. The number of clones studied per situation is given in the right column. ND, not done.

compared with 4/10 after culture with antiserum to the homologous peptide ($P = 0.002$). Similar experiments using isolate SP2-PI and the homologous rabbit antiserum A-SP2V3N2 did not lead to the emergence of variants with changes in the target N-terminal sequence of V3. Instead, variants with an aspartic acid instead of a glutamic acid at position 325 (E $\rightarrow$ D) in the C-terminal half of the V3 loop appeared (frequency 0/50 before, and 10/10 after serum exposure. $P < 0.0001$). Position 325 is eight residues away from the distal end of the immunising peptide SP2V3N2. A substitution at position 325 also occurred when SP2-PI was exposed to neutralising autologous serum SP2-E. Notably, serum SP2-E contained abundant IgG binding to two out of three autologous N-terminal peptides (Fig. 1b). The results show that neutralisation via the N-terminal half of the V3 loop can lead to selection of variants which have substitutions within the target sequence or at remote residues in the C-terminal half of the V3 loop.

DISCUSSION

Although several researchers have emphasised previously the importance of anti-V3 antibody responses in slowing disease progression [Fenouillet et al., 1995; Yamanako et al., 1997], some contradictory reports are

TABLE III. Neutralisation of Primary Isolates SP1-PI, SP2-PI, FP1-PI, and FP2-PI by V3 Specific Rabbit Antisera*

Isolate	Infectious dose ($TCID_{50}$)	A-SP1V3N2 INCTRPNKNTRKSIPIGPG	A-FP2V3N2 INCTRPNNTRKSIRIQRGPG	A-FP1V3N2 INCTRPSNTRRGIHMGPG	A-SP2V3N2 INCTRPNNKTRKSINIGPG	A-SP2V3N3 INCTRPNNTRKRMTMGPG	A-SSC INCTRPNNTRKSIHIGPG
SP1-PI	15	200	0	50	200	800	0
FP2-PI	20	0	200	0	100	100	200
FP1-PI	56	50	50	800	50	100	100
SP2-PI	18	100	50	100	200	0	0

*Neutralisation assays using rabbit antisera to single V3 peptides tested against four primary isolates. Antiserum name is given at the top of a column together with the amino acid sequence of the respective peptide immunogen. Immunising peptide name can be deduced by removing the prefix "A" from the antiserum name (cf. figure 1). Homologous combinations are boxed. A-SSC is the antiserum to a south Swedish consensus V3 peptide.

also available [Hogervost et al., 1995]. In this in-depth study of four patients who lacked IgG binding to autologous V3 peptides, general conclusions are not drawn on the correlation between IgG binding to V3 peptides and disease progression. However, some pertinent observations are discussed. IgG binding to several V3 peptides decreased and was lost prior to the development of AIDS in one patient, and there was relative abundance of such peptide-binding IgG in sera from the slow progressors compared with that detected in the fast progressors. There are reports of defects in the anti-V3 IgG repertoire of patients infected with HIV which develop with disease progression [Schreiber et al., 1994, 1997a,b; Lawoko et al., 1995]. They could explain the decrease over time in titres of anti-V3 IgG [Fenouillet et al., 1995; Yamanako et al., 1997]. A number of observations therefore, are consistent with an important role for V3 in slowing disease progression.

Even though sera from all four patients lacked IgG binding to at least one autologous V3 peptide, they nonetheless neutralised both autologous (three out of four cases) and heterologous (in all cases) primary isolates. This is not surprising since neutralisation of HIV-1 can be mediated via epitopes both within and outside the V3 loop [see, e.g., Wyatt et al., 1998]. In one report, the depletion of anti-V3 IgG from neutralising sera did not affect primary HIV-1 isolate neutralising capacity [Vancott et al., 1995]. Consequently, it was suggested that the major determinants of primary isolate neutralisation, unlike those of serially passaged virus, lie outside of the V3 domain. Considering that some sera contain selective defects in the anti-V3 IgG repertoire however, the use of one or a few V3 peptides in depletion experiments might not rid the sera of all anti-V3 antibody. Second, anti-V3 mediated neutralisation by such sera may already be compromised, as demonstrated in this paper, to the extent that further removal of V3 antibody will not diminish its neutralising titre. Several kinds of anti-V3 antibodies are induced in HIV infection. Some are capable of binding peptides in solid-phase assays, while others only bind to the V3 in native gp120, prompting suggestions that many V3 epitopes are conformational [Moore, 1993; Moore et al., 1994]. Antibodies that only bind the V3 loops of native gp120 are probably directed at conformational epitopes [Schreiber et al., 1997], while the linear epitopes can be mimicked by short synthetic peptides and used for instance, in serological subtyping of HIV-1 [Cheingsong-Popov et al., 1998]. Consequently, if the different conformations present different epitopes, then the loss of respective antibody reactivities may occur independently.

All V3 peptides to which autologous IgG binding were not detected by EIA were found to be immunogenic in rabbits and induced antibodies that were broadly neutralising, and broadly peptide binding. The respective human hosts must, therefore, have selective defects in their recognition of the homologous V3 epitopes in vivo, as mimicked by N-terminal V3 peptides. Disregarding the role of neutralising epitopes outside

the V3 loop, variants of isolates SP1-PI and FP2-PI replicating after 90% in vitro autologous serum neutralisation carried V3 sequences to which the sera lacked IgG in the peptide binding assay. In the case of SP1-PI, the selected variant contained a proline residue at position 313, found earlier to abrogate IgG binding to V3 peptides. Thus, our in vitro experiments with primary isolates suggest that anti-V3 antibodies play an important role in the in vivo selection of neutralisation resistant viruses. In line with this, patient FP1 lacked IgG binding to all V3 peptides and also lacked autologous neutralising activity. At the same time FP1 had IgG that neutralised heterologous primary isolates suggesting that neutralising antibodies to epitopes outside the V3 domain were operating. In the fourth isolate (SP2-PI), the main variant replicating in the culture exposed to neutralising autologous serum represented a minor variant in isolate SP2-PI cultured without serum, indicating that the serum exerted selection pressure. The selected V3 sequence in this case contained a substitution (E $\rightarrow$ G at position 325) in the C-terminal half of the loop. Conceivably, such a change could alter the conformation of the V3 loop through interactions between the amino and carboxy termini within a β-hairpin overall structure [Zvi et al., 1997]. The presence of conformational neutralisation epitopes in the V3 loop, as well as the changes associated with neutralisation escape in pre-immunised chimpanzees inoculated with the IIIB strain of HIV-1 have been reported previously [Nara et al., 1990, 1992]. The conformation of an 18-mer V3 peptide of HIV-1_{IIIB} bound to the Fab fragment of an anti-gp120 HIV-1 neutralising antibody was determined using NMR spectroscopy. The peptide forms a 10 residue loop, which includes the crown motifs, while two segments flanking either side of it interact extensively in a β-sheet conformation [Zvi et al., 1997]. One can imagine therefore, that mutations in either the proximal or distal β-strand would disturb their intricate interaction and cause conformational change.

Since the antisera we worked with lacked IgG binding to autologous V3 peptides, it was interesting to determine what V3 variants would be selected by neutralising rabbit antisera directed specifically against the same peptides. Culture of isolate SP1-PI in such antiserum resulted in the selection of a variant containing a substitution within the N-terminal antibody target sequence (G $\rightarrow$ R at position 315). Neutralisation of a second isolate (SP2-PI) by V3-specific rabbit antiserum selected for a variant with a substitution outside the antibody target sequence (E $\rightarrow$ D at position 325). In experiments using a molecular clone of HIV-1 and a neutralising monoclonal antibody directed at an epitope in the V3 loop, McKeating et al (1989) demonstrated that neutralisation escape resulted from an amino acid substitution within the antibody binding site. Our work goes further in demonstrating that escape from V3 mediated neutralisation of a primary isolate can result from amino acid substitutions either in the vicinity of the antibody binding site, or distal to it, in both cases within the V3 loop. Reportedly, positions 313 and 325 are among several at which V3 loop variation is concentrated in vivo [Milich et al., 1997; Yamaguchi et al., 1998; Poss et al., 1998]. Since position 325 is also a known determinant of virus cell tropism [Milich et al., 1997], functional restriction of V3 variation is implicated.

To summarise, V3 peptides to which autologous IgG binding was lacking represent structures that are both antigenic and immunogenic. The epitopes concerned can confer neutralisation of primary isolates. Despite lacking IgG binding to autologous V3 peptides, the human sera neutralised both autologous and heterologous isolates. In vitro exposure of patient primary isolates to autologous neutralising serum, or to V3 specific rabbit antiserum selected for variants with amino acid changes within, or outside the antibody target sequence, at sites which are known to vary successively in individuals with natural infection.

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